REVIEW OF LITERATURE
2.1. Kidney stone disease

It is estimated that at least 10-15 per cent of the population in the industrialized part of the world is afflicted by urinary tract stone disease. In some geographical areas the prevalence is even greater, for instance, in the Arabian countries. Therefore, it appears to be a changing pattern in as much as stone disease now is becoming more common in young women (Robertson et al. 2000). The formation of urolithiasis occurs in both men and women but the risk is generally higher in men (Davidson et al. 2009). With its multifactor etiology and high rate of recurrences, urinary tract stone disease provides a medical challenge. It is well established that calculi in the urinary tract occur more frequently in the natives of certain geographic regions of the world (e.g. Southern China, Northern India with some pockets in Assam and Manipur, certain regions of Turkey and Egypt, the Volga Valley in U.S.S.R., Fast coast of U.K. and South-Eastern States of U.S.A.) than others (Straffon and Higgins 1970). Since considerable variations exist in these so-called stone belts regarding the mineral content of the water and soil, dietary habits of their natives, and environmental conditions, it is safe to conclude that factors other than these may be important in the etiology of urolithiasis. For a group of first time calcium stone formers, the
expected risk of recurrent stone formation during a 10-year period was estimated to 30 percent and in those who had formed at least two stones at the start of follow-up the corresponding figure was as high as 70 percent (Tiselius 2000). For patients with certain other types of stone the recurrence risk might be even more.

2.2. Aspects on the process of stone formation

During the process of water conservation, kidneys supersaturate urine (Carvalho et al. 1999). Supersaturation (SS) is the driving force for crystallization in solutions like urine, which means that it will contain crystals that are formed spontaneously. If inhibitors of crystal formation were not able to act and control their size, the final result will be nephrolithiasis and/or nephrocalcinosis (Carvalho et al. 2002). The understanding of crystalluria requires some knowledge of crystal nucleation, growth and aggregation, all of which depend greatly on solution concentration. Both the monohydrate and dihydrate species of calcium oxalate (CaOx) crystals are present in kidney stones (Wesson et al. 1998). It has been proposed that crystalluria may be predictive of a nephrolithogenic tendency (Rushton and Spector 1982). Also, crystalluria with oxalate crystal volume measurement is a non-invasive, easily performed investigation, and can give feedback on the efficacy of urolithiasis therapy (Jouvet et al. 1998). In terms of calcium oxalate crystallization the situation has proved to be much more complex and the various parts of this process are schematically summarized in figure 2.1 the fundamental steps in stone formation are

a) Crystal nucleation
b) crystal growth

c) crystal aggregation

d) crystal retention

The last step in the process is poorly understood, but most certainly an assembling of crystalline material is necessary to build up a stone. Nucleation of calcium oxalate is assumed to be induced by one or several promoters. Growth and aggregation of calcium oxalate crystals can proceed as long as the ion-activity product of calcium oxalate exceeds the solubility product (SP).

All these processes are counteracted by inhibitors. The inhibition is accomplished by either large or small molecules. Whether the initial crystallization takes place as free or fixed particles has been a matter of debate over the years and it has generally been assumed that the precipitation of
Figure 2.1. Major steps in calcium salt crystallization.

oxalate is too slow to give crystals of sufficient size to be trapped in the tubular system unless there is some kind of fixation of the crystalline material (Finlayson and Reis 1978). Recalculation of old data has, however, indicated that free particles of calcium phosphate as well as of calcium oxalate might form at the levels of supersaturation that occasionally are built up in nephron urine (Kok and Khan 1994). In this way crystals might become large enough to be trapped intra-tubularly. Irrespective of whether the initial crystallization is the result of free or fixed particles, stone formation cannot occur unless crystal material is retained in the renal collecting system. Retention of crystal material can, however, also be the result of interaction between the crystals and the cells and such a mechanism is assumed to play an important role (Mandel 1994; Khan 1996; Verkoelen et al. 1995, Verkoelen et al. 1997; Lieske and Deganello 1999). It needs to be emphasized again that if the urine is not
sufficiently supersaturated, there will be no salt precipitation and accordingly, under such conditions, the fundamental prerequisite for stone formation is lacking. This important fact is the theoretical basis for most of the recurrence preventive treatment regimens that are used today clinically in patients with urinary tract stone disease. In as much as recurrence preventive treatment ideally should be as selective as possible, there are two steps that are necessary to enable such an action. First, to decide whether the patient has a high supersaturation level or may be at risk of forming critically supersaturated urine. Thereby the supersaturation or the ion-activity product is the effective or secondary risk factor. Second, it is necessary to identify those factors that in an important way contribute to the supersaturation and the risk of crystallization. These latter variables can thus be considered as primary risk factors.

There is evidence that the process of calcium stone formation starts as a precipitation of calcium phosphate either in the loop of Henle or in the distal part of the distal tubule (Lupták et al. 1994; Tiselius 1996; Asplin et al. 1996; Kok 1997; Höjgaard and Tiselius 1999). Although the urine at these levels of the nephron might be critically supersaturated with calcium oxalate in patients with hyperoxaluria and in experimental animals following administration of ethylene glycol, the ion-activity product of calcium oxalate is usually too low to result in calcium oxalate crystal formation (Lupták et al. 1994). Any crystallization that occurs in this part of the nephron most certainly is facilitated by promoters (Figure 2.2) and it has been suggested that lipoprotein membranes from the brush border of proximal tubular cells might serve this purpose (Khan et al. 2000). Experimental research has shown that the brush border membrane might be injured by free radicals formed as the result of toxic effects on the cell. This might lead to lipid peroxidation and cell death (Thamilselvan
and Khan 1998). The released membrane fragments that are transported down the nephron thereby can supply a suitable surface for deposition of both calcium oxalate and calcium phosphate. There are also other less specific constituents of nephron urine that have the capacity to induce nucleation of calcium salts, such as, for instance, blood cells, crystals of sodium urate, cholesterol, or other intratubular particles. Crystals of calcium phosphate that form at a high nephron level (Tiselius 1997) (Figure 2.2) might be dissolved when they are exposed to the acid urine in the collecting duct (Højgaard 1999).

Dissolution of calcium phosphate causes a high level of supersaturation with calcium oxalate by increased urine concentration of calcium. Nucleation of calcium oxalate can thus take place either by epitaxis on the surface of the dissolving calcium phosphate crystal or by nucleation, with or without the contribution of a promoter, or by nucleation in the macromolecular environment that surrounds the calcium phosphate crystals. The latter process might take place either freely in the tubular lumen or more likely at tubular wall.

Under normal conditions, the crystals of calcium oxalate and calcium phosphate that form are small and well protected from crystal growth and crystal aggregation by a cover of inhibitory macromolecules. The negatively charged macromolecules have a high affinity to the positively charged surface of calcium salt crystals. The aggregation inhibiting properties of small as well as of large molecules are related to their electronegativity which establishes repulsive forces between adjacent crystals and between the crystals and the similarly negatively charged macromolecular layer on the surface of tubular cells (Figure 2.3).
Figure 2.2. Overview of the various possible steps in calcium stone formation. (SS=supersaturation)
Figure 2.3. (a) Hypothetical interpretation of the possible series of events of the normal crystallization in urine: (1) brush-border membrane of proximal tubular cells; (2) repulsion between small calcium phosphate crystals; (3) between calcium phosphate crystals and tubular cells; (4) elimination of small calcium phosphate crystals by dissolution or passage with urine; (5) internalization and intracellular dissolution of calcium phosphate crystals; (6) primary nucleation of calcium oxalate; (7) calcium oxalate nucleation induced by calcium phosphate; (8) attachment of small calcium oxalate crystals to the tubular cell; (9) internalization and dissolution; (10) macrophage destruction of calcium oxalate crystals in the interstitial tissue, small intraluminal crystals of calcium oxalate are excreted with urine.
Figure 2.3. (b) Hypothetical interpretation of the possible series of events of the *pathological crystallization* in urine: (1) destruction of the brush-border membrane of proximal tubular cells; (2) nucleation of calcium phosphate crystals promoted by membrane fragments; (3) formation of large masses of calcium phosphate crystals by growth and aggregation; (4) adherence of crystal aggregates to the tubular surface; (5) dissolution of calcium phosphate in acid urine and nucleation of calcium oxalate; (6) formation of a large mass of calcium oxalate and calcium phosphate crystals attached to the tubular wall; (7) primary nucleation of calcium oxalate induced by membrane fragments with or without participation of urinary macromolecules; (8) attachment to the tubular cell of large calcium oxalate aggregates; (9) partial dissolution of internalized calcium oxalate crystal material; (10) migration of crystals to the interstitial tissue where the capacity of macrophages is insufficient to cope with the large crystal masses; (11) destruction of tubular cells with binding of crystals to the basolateral membrane; (12) formation of an intratubular stone nidus by an assembling and trapping of crystal aggregates; (13) interstitial migration of crystalline material to the papillary tip.

In this way, it is likely that small crystals are fast and can easily move through the tubular system and be excreted with urine. It is possible that small calcium phosphate crystals are completely dissolved during their transport through the collecting duct. Under
appropriate conditions primary nucleation of calcium oxalate might occur in collecting duct urine. As long as these crystals remain small and are well protected from growth and aggregation they leave the tubular system without problems. The interaction between the tubular cells and crystals are modified by several macromolecules excreted with urine or secreted by the tubular cells (Verkoelen et al. 1997; Lieske et al. 1997c).

Experimental studies with cell cultures have shown that calcium oxalate monohydrate crystals adhere to tubular cells in a specific and rapid way (Lieske and Coe 1996). The cellular affinity for brushite (Bru) crystals was much less pronounced. It has moreover, been shown that a number of polyanions might prevent the adherence of crystals to cells. Such an effect accordingly was recorded for glycosaminoglycans (heparin, heparan sulfate, hyaluronic acid, and chondroitin sulfate), citrate, nephrocalcin, and uropontin. Tamm–Horsfall protein (THP) on the other hand, did not counteract the adherence of crystals to the cells, but inhibited crystal endocytosis. It was concluded that crystals of calcium oxalate binds to sialic acid residues on cell surface glycoproteins. Also, the lipids of the plasma membrane appear to be of great importance for the adherence of crystals (Bigelow et al. 1997).

Normally, cells lining the tubular system are well protected from crystal adherence, but the situation alters dramatically following cell injury. The same principle for crystal attachment as for calcium oxalate is applicable to crystals of hydroxyapatite (HAP) (Lieske et al. 1997b). Crystals of calcium oxalate monohydrate (COM), calcium oxalate dihydrate (COD), and HAP that have adhered to the cell surface might be internalized by endocytosis (Lieske et al. 1992; Lieske et al. 1999). Some of these crystals might be dissolved by the action of lysosomal enzymes within the tubular cell, whereas others might be transported to
the basolateral membrane and expelled into the interstitial tissue where macrophages and other inflammatory cells can take care of the crystals and destroy them (Khan 1996). In this way a substantial amount of crystalline material might be removed from the tubular system and it is likely that this is one of the defence systems that the kidney has developed to protect from intratubular crystallization and obstruction. In response to high concentrations of oxalate or calcium oxalate crystals tubular cells might proliferate and thus increase the capacity to eliminate crystals. The risk of crystal adherence is certainly greatest for calcium phosphate and calcium oxalate crystals that are large and thus move slowly through the nephron. For very large crystals and crystal masses the repulsive forces described above are probably insufficient to counteract both crystal aggregation and crystal–cell adherence. It is also well recognized that patients with calcium stone disease excrete in their urine larger crystals and crystal aggregates than normal subjects. Given these principles for the normal crystallization in the nephron, a pathological crystallization leading to stone formation might be the net result of one or several abnormalities or defects in the control of this process (Figure 2.3). Low concentrations or structural abnormalities of crystallization modifying macromolecules or small molecules will cause increased growth and aggregation of crystals so that large crystal masses form either of calcium phosphate or of calcium oxalate (Coe and Parks 1990). Large crystal masses with or without insufficient protection by macromolecules will adhere to the tubular cell. The crystals might alter the plasma membrane so that endocytosis occurs whereas crystals of reasonable size can be taken care of and destroyed by the cell, larger crystal agglomerates might cause cell death (Khan et al. 2000; Koul et al. 1996). When the crystals that are bound to the basolateral membrane or deposited interstitially are too large, the capacity of macrophages and inflammatory cells
will probably be insufficient to cope with the crystals. Such crystal material might accordingly be transported within the interstitial tissue down to the papilla where it can provide a basis for crystal deposition and growth following erosion of the epithelial surface. It is understood that the insufficient or defect control of the crystallization process also will result in development of large crystals or crystal agglomerates that remain within the tubular lumen. Under such conditions crystals of calcium oxalate or calcium phosphate might be trapped at the lower and narrow end of the collecting duct and thereby serve as a nidus for further crystal deposition in the supersaturated urine. Figure 2.4 summarizes the various possibilities of calcium salt crystalluria and calcium stone formation. Small crystals of calcium oxalate or calcium phosphate that might have formed in the nephron can disappear either by intraluminal dissolution or by cellular action. These crystals can also be excreted with urine as microscopic crystalluria, which is a common finding in both stone formers and normal subjects.

A primary precipitation of calcium phosphate and subsequent dissolution in acid collecting duct urine can give rise to crystal aggregates containing calcium oxalate and calcium phosphate or, in case of complete dissolution, pure calcium oxalate.
Figure 2.4. Possible outcomes of calcium salt crystallization in the urinary tract. Small crystals of calcium phosphate or calcium oxalate can disappear by dissolution (1, 9) or remain small (3, 8). Nucleation of calcium oxalate might be induced by calcium phosphate (2, 6, 7). Following complete dissolution of calcium phosphate pure calcium oxalate aggregates can form (7). In constantly alkaline urine there is no precipitation of calcium oxalate and the result will be pure calcium phosphate aggregates, either in the form of hydroxyapatite (4) or brushite (5). A primary nucleation of calcium oxalate results in small crystals (8) or crystal aggregates (10).

Pure calcium oxalate aggregates also might form when calcium oxalate is primarily precipitated in the nephron unrelated to a calcium phosphate crystal phase (Lieske et al. 1998). In patients with a constantly high pH, calcium phosphate crystals will not dissolve
and inasmuch as calcium phosphate is the favoured crystal phase in alkaline urine, pure calcium phosphate crystals and stones will be the result. Such crystals most commonly consist of HAP, but might occasionally be composed of Bru, particularly at a lower pH.

2.3. Epidemiology of renal stones

Urolithiasis is a clinical disorder which is known to be caused by multiple etiologic factors. These could be prurinary and urinary risk factors leading to metabolic disorders. The prurinary factors could be both intrinsic and extrinsic.

2.3.1. Prurinary risk factors

A. Intrinsic factors

a) Heredity – Several workers have given evidence to support the concept that the familial incidence of urinary calculi is related to heredity. Now it is known to be a polygenic defect influencing various enzymes of oxalate, uric acid metabolism (Khan and Canales 2009). Ethnicity has also been shown to be an important factor for calculosis. For example, a low incidence of calculi has been found in Negroes of Africa and America, North American Indians, natives of Israel, South Indians and South Americans.

b) Age - The maximum incidence of idiopathic calcium stone disease has been shown to occur between the third and the fifth decade of life. It has been found to be quite uncommon in children and elderly people (Soucie et al. 1994; Hiatt and Friedman 1982).

c) Sex – Males are generally known to be more prone to calcium stone disease as compared to females. This could be due to the fact that as compared to females, males are known to excrete more calcium, oxalate, uric acid in their urine leading to its higher saturation. The
anatomical structure of the female urogenital tract also facilitates easy passage of initially formed crystalloid. Moreover, effect of estrogens in increasing the urinary citrate excretion which have a solubilising effect on calcium could be responsible for the low incidence of urinary calculi in females. The incidence of renal calculi is four times higher in males as compared to females (Davidson et al. 2009; Hiatt and Friedman 1982).

B. Extrinsic factors

a) Geographical distribution- The incidence pattern of renal stones in India has been determined and two high incidence stone belts have been found to occur (Coblabawala 1971). The first belt starts from Amritsar in North and while passing through Delhi and Agra ends up in U. P. The other belt which starts from Jamnagar in the West Coast extends inward towards in Jabalpur in Central India. Very low incidence has been found in West Bengal and coastal areas of Maharashtra, Karnataka, Kerala, Tamilnadu and Andhra Pradesh.

The Northern part of the United States (except in Negroes) has also been shown to have a relatively high incidence of urinary calculi. Other geographic areas surveyed and reported to have high incidence include Central Europe, Japan, North India, Pakistan, Northern Australia, parts of Malaysia and China (Zerwekh et al. 1983).

b) Climatic factors – The high summer temperatures in South Eastern United States have been shown to be responsible for high incidence of urinary calculi. Reasons for higher incidence in summers could be an increased conversion of vitamin D to its active metabolites resulting in increased calcium absorption from intestines. Decrease in urine production due to greater loss of water from sweat causes an increase in the concentration of
stone constituents in urine and hence supersaturation of urine with stone forming constituents leading to calculosis (Milliner 1995).

c) Dietary factors – The dietary content of animal proteins and fats has been found to be approximately 5 times higher and that of sugar 10 times greater in developed countries (Milliner 1995) than in Africa (South of Sahara) where occurrence of Ca-stones is very rare. Use of refined carbohydrates and animal proteins has further been shown to increase urinary calcium and oxalate excretion which could be responsible for high incidence of renal calculi.

Sucrose has been observed to cause a significant increase in intestinal calcium absorption in idiopathic stone formers as well as in normal subjects. The effect of nutrient rich and fiber depleted diets on absorptive and excretory mechanism is likely to increase urinary supersaturation with respect to CaOx (Taylor et al. 2009).

Vitamin K deficiency is known to be associated with stones of renal origin. Vitamin K is known to promote the formation of gamma-carboxy glutamic acid which has high affinity for Calcium. If the renal carboxy glutamic proteins were to play a role in tubular handling then a decrease in their formation as possibly reflected by the decrease in urinary gamma-carboxy glutamic acid excretion in vitamin K depleted animals should lead to an altered tubular handling at calcium (Angayarkanni and Selvam 1998). Thiamine deficiency has also been reported to increase the risk of calcium lithiasis by causing hyperoxaluria and/or decreasing the citrate excretion in urine.

d) Occupation- It has been found that urinary calculi are more likely to be found in individuals having sedentary occupations as compared to individuals doing manual work
(Taylor et al. 2009). The possibility exists that the physically strenuous nature of the manual work leads to the disruption of the crystal aggregation phenomenon in the urinary tract.

e) Water intake – A number of investigators have shown that increased water intake and increased urinary output decreases the incidence of urinary calculi (Miggiano and Migneco 2007). A small urinary volume, whether caused by low fluid intake or increased fluid loss through other routes has been shown to result in increased stone formation.

2.3.2. Urinary factors

a) Effect of pH- Uric acid stones occur in patients with very low urine pH (below pH 5) and in those with hyperuricosuria (Sakhee et al. 2002). In some patients low urine pH is caused by a defect in renal ammonia secretion that results in less buffering of secreted hydrogen ion and lower urine pH.

b) Concentration of salts in urine - The key process in the development of kidney stones is supersaturation. Salts such as calcium oxalate, uric acid, cystine, or xanthine can become extremely concentrated under certain circumstances. If the volume of the urine is significantly reduced; or if abnormally high amounts of crystal forming salts are present, they precipitate out and form crystals.

c) Volume of urine – Increasing urinary volume is an important tool in the prevention of calcium renal stones. Urine dilution considerably reduces crystallization phenomena induced in vitro by an oxalate load in both calcium stone-formers and normal subjects (Guerra et al. 2005).
d) Enzymes – The initial step in the pathogenesis of urolithiasis must be the precipitation of an organic matrix of mucoproteins. An important factor in this process may be the activity of and/ or concentration of the urinary enzyme, urokinase, which would affect the level of urinary mucoproteins. A decrease has been observed in urinary urokinase concentration of renal stone patients which, once again, underlines the possible involvement of urokinase in renal stone formation (du Toit et al. 1997).

2.4. STONE & STONE MATRIX

Like other products of crystallization in biological systems (Lowenstam and Weiner 1989), stones are a composite of crystals and organic material, often referred to as matrix (Royce 1977; Iwata et al. 1988; Khan and Hackett 1993). A variety of crystals including CaOx, CaP, uric acid, struvite, and cystine can be present in stone (Finlayson 1978; Finlayson 1977; Khan and Hackett 1987). CaP crystals appear most frequently in both the urine and stones however, CaOx is the major crystal in most stones. Stones, particularly those containing CaOx or uric acid have a compact structure. Their outer surfaces appear smooth at low magnification but reveal the presence of individual tabular or plate-like CaOx monohydrate (COM) crystals at higher magnifications. Crystal habits are generally not evident on surfaces exposed by cutting or fragmenting the stone. Such surfaces are typically stratified with radial striations and concentric laminations or layers, with radial striations being the predominant feature. Some of the striations run through many laminations while others are limited to only one. Many converge to a point at the base of a lamination
mimicking the arrangement of petals in a flower. These points are suggested to be the nucleation sites of crystals.

The laminations are approximately 50-60 μm thick and in many stones can be easily separated from each other exposing the underlying surfaces. The latter show the same structure as the outer stone surface, with protruding tips of the tabular COM crystals frequently covered with amorphous to flaky matrix material. Overall it is a highly ordered structure. Many stones have a well-defined nucleus that is less ordered, with a granular and non-stratified appearance. It is generally occupied by spherulitic or amorphous CaP and/or aggregates of dumbbell shaped twinned COM crystals. CaP frequently fills the space between CaOx crystals as well as that in the concentric laminations.

The organic matrix of most urinary stones accounts for 2-3% of their total dry weight. Matrix consists of macromolecules generally present in the urine. These molecules play a significant role in the development of kidney stones. Some of them promote crystal formation, growth, aggregation and retention, while others inhibit these processes. Their activity is often complex and depends on the urine conditions prevailing at the time of crystallization or retention. The same macromolecule can both promote and inhibit a process. For example macromolecules behave differently in solution than when they are attached or adsorbed to a surface. It may well be, that compounds free in solution cover a crystal surface and inhibit its growth or ability to aggregate while the same compound bound to a surface acts to accumulate salt ions and forms a template for the first nucleus. The latter will play a role when stone formation involves processes at cell surfaces and in the sub-epithelial space (Kok and Schell-Feith 1999).
Boyce et al. defined and established the importance of stone matrix in urolithiasis, proposing that the matrix actively participates in the assembly of kidney stones (Xie et al. 2001). In their view, the matrix acts as a template and controls crystallization within its bounds. An opposite hypothesis was advanced by Vermeulen et al. who viewed the matrix and its ubiquitous presence as merely coincidental because stones form by crystallization in urine in the presence of large macromolecules (Vermeulen and Lyon 1956; Finlayson et al. 1961). According to them the matrix is adventitiously acquired, primarily by physical adsorption of urinary mucoproteins on crystal surfaces. Another hypothesis proposed by Khan et al explains that the role of matrix compounds is different in the formation of the stone center and in the subsequent build-up of the stone. The first is a short-term event involving crystal formation and retention. The second is a long term event occurring after a stone nidus has been formed and retained. Both events do not necessarily take place at the same site. Solution depletion (Leal and Finlayson 1977) and examination of crystals incubated in protein solutions by transmission electron microscopy (Khan et al. 1983) tested the theory of physical adsorption of urine proteins on surfaces of CaOx crystals. Results showed proteins have a strong affinity for CaOx crystals. Adsorption of anionic proteins was sensitive to calcium ion concentration, whereas cationic protein adsorption depended upon the oxalate ion concentration with temperature and pH playing only a minor role. Proteins formed a discontinuous coat around the crystals ranging in thickness from 10 to 20nm. It has been suggested that newly formed crystals with a macromolecular coat are less likely to dissolve during the routine urinary ionic and pH changes and therein may lie the importance of matrix in stone formation (Khan and Hackett 1993).
2.4.1. Morphology of renal stone

Morphological examination of decalcified and intact stones shows that matrix is pervasive, distributed throughout the stones, and has amorphous and fibrous components (Boyce 1972; Iwata et al. 1988; Khan et al. 1988). The crystal matrix association is so intimate that the dipyramidal habit of CaOx dihydrate [COD], the monoclinic or plate-like habit of COM and spherulitic habit of CaP stay intact after total removal of the mineral content (Khan and Hackett 1986). Scanning electron microscopic examination of decalcified COM stones showed the matrix to be organized in concentric layers of 2-5 μm thick. The thickness was uniform throughout the circumference and smaller than the laminations seen in an intact stone. Successive layers appeared as rings of an onion bulb with little or no space in between. They consisted of loosely or tightly matted fibers and contained empty columns representing crystal ghosts presumably formed by dissolution of tabular COM crystals. Crystal ghosts were often arranged radially in relation to the stone centre, reminiscent of the radially arranged crystals in the intact stones.

Examination of the concentric layers by transmission electron microscopy confirmed that they contained radially arranged columnar crystal ghosts surrounded by an amorphous electron dense coat and embedded in a fibrous matrix. Electron dense material was also found inside crystal ghosts. Cellular degradation products including degenerating nuclei, mitochondria, endoplasmic reticulum and membrane fragments as well as vesicles occupied the intercrystalline spaces (Khan and Hackett 1993). The layered matrix was sudanophilic (Khan et al. 1988) and stained positive with periodic acid Schiff (PAS) and colloidal iron (Khan and Hackett 1986), indicating the presence of lipids, glycosaminoglycans (GAG) and proteins. Examination of decalcified stones using antibodies against osteopontin (OPN) and
calprotectin showed them to stain both the stone center and concentric laminations (Tawada et al. 1999). OPN was detected both inside the crystals as well as on their surfaces. Ultrastructural examination of decalcified stones also showed the crystal associated matrix to stain positive with malachite green indicating the presence of phospholipids (Khan et al. 1996).

### 2.4.2. Chemical Composition

The organic matrix of urinary stones contains lipids, GAG's (20%), carbohydrates and proteins. Proteins comprise approximately 64% of the matrix. Table 2.1 lists the compounds, which have been identified in matrices of urinary stones. Most of them are proteinaceous in nature. A number of other proteins have also been detected but not identified. Initially lipids were not recognized as constituent of stone matrix (Boyce 1972) even though detected as an osmiophilic substance during histochemical examination of decalcified stones. All urine macromolecules can become part of stone matrix but only some are there because they have participated in crystallization and stone formation. This appreciation led investigators to study crystallization *in vitro*; using freshly collected urine to determine the macromolecules that become a part of the crystal matrix (Morse and Resnick 1989).

### 2.4.2.1. Glycosaminoglycans (GAGs)

Heparan sulphate (HS) and hyaluronic acid (HA) are the two major GAGs in the matrix of both stones and CaOx crystals formed in urine (Roberts and Resnick 1986; Yamaguchi et al. 1993). GAGs can account for up to 20% of the stone matrix (Nishio et al.
The most abundant urine GAG, chondroitin sulphate (CS) was not found in these matrices indicating selective incorporation (Suzuki et al. 1994a). Keratan sulphate and dermatan sulphates are present in trace amounts.

### 2.4.2.2. Proteins

More than twenty individual proteins have been detected in the matrix of various types of stones. While most of them have been identified (Table 2.1), few are fully characterized and some still remain nameless (Binette et al. 1996) and a few await confirmation of their identity (Tang et al. 1995). There are Human serum albumin (HAS), α and γ-globulin and Tamm-Horsfall Protein (THP) were the first proteins identified in stone matrix (Boyce et al. 1962).

Albumin is a major component of the matrix of all stone types including CaOx, uric acid, struvite and cystine. It is also found in the matrix of CaOx and CaP crystals precipitated from human urine and it is more pronounced in crystals induced in stone formers urine (Atmani et al. 1998; Atmani and Khan 2002).

Both CaOx and CaP crystals are known to adsorb HAS. THP is not always detected in stones and even then in only minor quantities, 0.002-1.04 mg/g (w/w) of stone (Grant et al. 1973).

**Table 2.1. Proteins, GAGs, lipids and small molecule detected in CaOx/CaP stones and/or crystals.**

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<td>α-1-microglobulin</td>
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<td>Superoxide dismutase</td>
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<td>18</td>
<td>Tamm-Horsfall Protein</td>
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<td>α and γ globulins</td>
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<td>Transferin</td>
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THP associated with CaOx crystals is easily removed by washing the crystals with sodium hydroxide solution (Maslamani et al. 2000; Gokhale et al. 1996a) indicating THP’s loose interaction with the CaOx crystal surfaces. Ultrastructural investigations of human CaOx urinary stones and CaOx nephroliths induced in an animal model supported the hypothesis that THP is not included in the crystals (Gokhale et al. 1996a Gokhale et al.1996b). This may explain THP’s scanty presence in the stone matrix. Of the other proteins listed in Table 2.1, osteopontin (OPN), α-1- microglobulin, urinary prothrombin fragment-1 (UPFT-1), and light and heavy chains of inter-α-inhibitor have been identified in the matrix of CaOx and CaP crystals precipitated from the urine of normal and stone forming individuals (Atmani and Khan 2002). Ultrastructural examination reveals OPN to be pervasive in the crystals and stones and a key component of the matrix of CaOx stones (Mckee et al. 1994; Hoyer 1994).
More OPN is present in CaOx monohydrate stones (800 µg/100mg stone) than in COD stones (10 µg/100mg stone).

2.5. MODULATORS OF CRYSTAL FORMATION AND RETENTION

In urine, three classes of modulators are recognized; low MW compounds like citrate and pyrophosphate, glyco-proteins, high MW non-protein compounds like acid mucopolysaccharides, glycosaminoglycans and various types of lipids. They modulate crystal formation and retention in the urinary tract either directly by interacting with the crystal or indirectly by influencing the urinary environment. From crystallization experiments with urine, it appears that in non-stone formers the concerted actions of these compounds ensure that:

1. The crystals formed remain unaggregated and small enough to be excreted (Kok et al. 1986a; Kok et al. 1990);

2. The crystals have a reduced affinity for epithelial cells (Verkoelen et al. 1996; Schepers et al. 2002; Licske et al. 1995) and;

3. The crystals if needed are easily recognized and removed by macrophages (de Water et al. 2001).

Crystallization in confined spaces, e.g. simulating Randall's plaque formation at the basal membrane below the tubular epithelium has been less studied but even here, inhibitors can decrease crystal growth rates (Achilles et al 1991; Achilles et al 1995). Which inhibitors are the most effective? The first approach to answering this question has been to identify individual urine compounds and test their "inhibitory" potency in crystallization and
cell-culture systems. The next problem has been to translate these data to the whole urine situation where singular inhibitors may also co-operate or compete with each other and where restrictions posed by the kidney and urinary tract itself (flow-rates, residence time and changing urine composition) affect their inhibitory and stimulatory powers. Undiluted whole urine strongly affects calcium salt nucleation, crystal growth and crystal aggregation. When preformed CaOx crystals were added to supersaturated whole undiluted urine their growth was almost completely stopped. Crystal growth only occurred when the supersaturation was drastically increased by adding extra oxalate (Kok 1996). Urine has an overabundance of inhibitors. Tested in vitro as single compounds some are clearly more effective than the others, however, experimental data suggest that when the most efficient compounds are lacking, others readily take over. For instance, the low MW compound citrate can inhibit crystal growth very effectively at concentrations between 0.1mM and 1 mM. When citrate was added at these concentrations to urine, however, it did not change the growth inhibitory action of that urine (Kok et al.1986b). In studies of large groups of stone formers and healthy controls where urine was tested in a 1:5 dilution, approximating the degree of dilution existing in the collecting ducts, both urine from stone formers and normal subjects strongly inhibited CaOx crystal growth (Kok et al.1986a; Kok et al. 1990; Erwin et al.1994). When all macromolecules were removed by ultrafiltration, the degree of crystal growth inhibition was only slightly reduced (Drach et al. 1979). In vitro tests have confirmed that macromolecules are the most effective inhibitors of crystal growth. Apparently the low MW compounds take over the inhibitory function when the high MW compounds are gone. An additional effect of crystal growth inhibition may be that supersaturation will persist longer and the process of nucleation will have more time to proceed (Erwin et al.1994). How
relevant this is, in view of the short transit times of urine through the nephron (a few minutes) (Kok and Khan 1994), is not clear. Normal urine can also strongly inhibit crystal aggregation. This function is reduced in single stone former urine and severely reduced in recurrent stone former urine (Kok et al. 1986; Kok et al. 1990). Aggregation is important as it can lead to particle retention, just like crystal cell interactions and disturbed flow conditions (Kok and Khan 1994). The inhibition of aggregation in urine is correlated to the citrate concentration (Kok et al. 1990). However, in ultrafiltered urine this relationship is gone (Koide et al 1981). Apparently citrate modulates the effect that high MW compounds have on crystal aggregation. In addition, it was found that the urinary macromolecular fraction (>10,000 D MW) of single stone formers inhibited crystal aggregation less than that of normals and even less by those from recurrent stone formers (Erwin et al. 1994). In this study 70-90% of the inhibitory activity was destroyed by proteinase treatment. Citrate has been shown to improve the inhibitory effect of THP on crystal aggregation (Hess et al. 2000). Overall it appears that urine contains numerous components, both small and large that competes and cooperates in modulating crystallization and inhibiting stone formation.

2.5.1. Low Molecular Weight Compounds

2.5.1.1. Pyrophosphate and Bisphosphonate

Pyrophosphate is present in urine at concentrations of 15-100μM. In a seeded crystal growth system, it inhibits COM crystal growth by 50% at 16-20 μM (Schwille et al. 1988; Kok et al. 1988; Ryall et al. 1988; Sidhu et al. 1986). It can also inhibit COM crystal growth
inside a gel matrix (Achilles et al. 1989) and effectively inhibits the growth of CaPs (Grases et al. 2000; Robertson 1973). If it is equally efficient in urine it can contribute 50% crystal COM growth inhibition in the collecting ducts (5 times dilution) and up to 80% in the urine. This efficacy prompted interest in therapies that raise the urine output of pyrophosphate and in non-biodegradable pyrophosphate analogues, bisphosphonates. These inhibit COM crystal growth at least as good as pyrophosphate, 50% inhibition at 1-20 μM concentrations (Kok 1995). Another feature of pyrophosphate, 2.0 10-4 mol/l, which it shares with citrate, 1.0 10-3 mol/l, is that COD is preferentially formed in its presence. The critical pyrophosphate concentration above which COD formation is prevented may be lowered to the physiological range by adding citrate (Yuzawa 1998). This is of interest as COD is the major crystal phase in normal crystalluria (Cerini et al. 1999) while there is more COM in stone former crystalluria and COM predominates in stones. The effects of pyrophosphate and bisphosphonates on crystal aggregation are more complex. Pyrophosphate increasingly inhibits crystal aggregation at increasing concentrations (Kok et al. 1988). At its concentration range in the collecting ducts it could contribute to the whole urine effect on aggregation. While some bisphosphonates have a comparable effect, others show no effect, a stimulatory effect on aggregation or even a biphasic effect, inhibiting aggregation at low concentrations and stimulating it at higher concentrations (Kok 1995). From experiments with a series of bisphosphonates where slight variations were made in their structure it was concluded that bisphosphonates bind to the crystal surface by a combined action of the two phosphonate groups and side chains in close proximity. Increasing the affinity for calcium of these side groups increased the capacity to inhibit crystal growth. The presence of two calcium binding phosphonate groups makes bisphosphonates to likely form large
polynuclear complexes with calcium ions acting as a bridge (Bone et al. 1979). These complexes act as one macromolecular structure and inhibition of aggregation is reversed to stimulation (Kok 1995). The complexes bind to more than one crystal at the same time and act as a bridge (viscous binding). Viscous binding can also explain why some macromolecules may at the same time strongly inhibit crystal growth and strongly stimulate crystal aggregation (Kok et al. 1986). Bisphosphonates with a large side chain (steric hindrance) do not form such large complexes and do not show stimulation of crystal aggregation. Growth inhibition by bisphosphonates also depends on their protonation state, thus on the pH and its pKa-values. The triply deprotonated form, present when the pH surpasses the pKa3-value, is the most effective in inhibiting crystal growth. A pH-dependency is also found for pyrophosphate and citrate (Caudarella and Vescini 2009). In the urine pH range ionic species of pyrophosphate are PP$^{4-}$, HPP$^{3-}$ and H$_2$PP$^{2-}$. The first two adsorb onto COM crystals (Wagner and Finlayson 1978) and will predominate at higher pH values. Variation of the pKa3 value of a bisphosphonate increases its activity at low urine pH values and might reduce its anti bone resorptive capacity. It may be possible to construct a bisphosphonate that strongly inhibits CaOx crystal growth and crystal aggregation at the urine pH levels and does not interfere with bone resorption activity at the low pH levels existing under active osteoclasts. Since pyrophosphate is an effective inhibitor under non-urine conditions, several groups have investigated if stone formers have a low urine pyrophosphate excretion. Pyrophosphate enters the urine in the glomerular filtrate. The plasma concentration is 2-3 µM, of which 70-80% is ultrafiltrable. The urine excretion rate is variable. In male non-stone formers the concentration averages 20-40 µM, the 24hr excretion rate is 30-60 µmoles (range 15-98 µmoles). It is nevertheless possible that
increasing pyrophosphate excretion raises the growth inhibitory power of urine and as such is beneficial.

2.5.1.2. Citrate

Citrate inhibits COM crystal growth at concentrations above 0.1 mM (Hess et al. 2000), which is in the range of its concentration in the loop of Henle (Kok 1996). It is also inhibits crystal growth in a gel matrix (Achilles et al. 1989). Citrate may contribute to crystal growth inhibition at sites where other, macromolecular, modulators have not entered the fluid yet (Caudarella and Vescini 2009). Citrate also affects crystal aggregation, both in solution (Kok et al. 1988) and in a matrix situation (Achilles et al. 1997). Tested as single modulator present, citrate inhibits crystal aggregation at concentrations above 0.1mM (Kok et al. 1988), and thus could be active up to the loop of Henle. However, this data cannot be directly extrapolated to the whole urine situation. When whole urine is tested in 1:5 dilution (the Dilution State in the collecting duct) urine is found to strongly inhibit crystal aggregation, and there is a strong correlation with the urine citrate concentration (Kok et al. 1986; Kok et al. 1990). But when all macromolecules are removed and citrate remains, the urine loses most of its capacity to inhibit crystal aggregation and the relationship between crystal aggregation inhibition and citrate concentration is lost.

2.5.2. High MW Compounds

2.5.2.1. Glycosaminoglycans (GAGS)

In 1684 Anton von Heyde discovered the presence of a mucoprotein matrix in stone (Gruber 1934). Later, urine was found to contain many different anionic proteins and non-protein anions like (GAGS), RNA and acid mucopolysaccharides. Most prominent are the
GAGS, polyanionic compounds with varying MW of usually 18-40 kDa but up to 106 Da. GAGS can enter the urine by filtration, by release from the glomerular basement membrane (Pitcock et al. 1988) from the surface of the tubular epithelial lining and the urothelium further down the urinary tract, including the bladder (Edyvane et al. 1983). Well-known GAGS include heparin (not present in urine) and the urinary GAGS heparan sulfate (HS), chondroitin sulfate A B and C (CS-A, CS-B, CS-C) dermatan sulfate (DS), keratan sulfate (KS) and the non-sulfated hyaluronic acid (HA). Some, but not all urinary GAGS are found in crystals and stones (Morse and Resnick 1989; Nishio et al. 1985; Roberts and Resnick 1986; Yamaguchi et al. 1993; Suzuki et al. 1994a).

Although quantity does not appear to play a role, some data indicate that the quality of GAGS may vary. Urinary macromolecules and urine from children inhibit crystal aggregation better than urine of adults. The macromolecule fraction of pediatric urine contained more GAGs (Teller et al. 1962). GAGs from stone formers had an increased nucleation promoting activity but similar crystal growth inhibitory activity (Erturk et al. 2002). The first appeared related to a changed action of HA in stone formers (Gohel et al. 1992). However, CS of healthy individuals also showed a basal crystallization-promoting property (Shum et al. 1999). Under inorganic solutions and urine conditions the non-urine GAG heparin is the most effective on a molar basis. Of the GAGs present in urine HS is most effective followed at a distance by CS and HA. The heparin analog pentosan polysulfate has an efficacy between heparin and CS. With respect to crystal cell interactions, coating of crystals by GAGs decreased the binding of crystals to renal epithelial cells in culture, but did not completely abolish it (Schepers et al. 2002; Lieske et al. 1995; de Water et al. 2001).
2.5.2.2. Proteins

2.5.2.2.1. Tamm-Horsfall Protein

THP was first isolated from the urine by Tamm and Horsfall and characterised as a glycoprotein that inhibits viral hemagglutination (Tamm and Horsfall 1950). It is one of the most abundant proteins in normal human urine and the major constituent of urinary casts. Muchmore and Decker isolated a protein called uromodulin from the urine of pregnant women (Muchmore and Decker 1985). Based on amino acid and carbohydrate analysis THP and uromodulin were shown to be identical (Kumar and Muchmore 1990). THP has a molecular weight of approximately 80 kDa with a tendency to aggregate to the polymeric form. Polymerisation is increased in the presence of free calcium ions, at high ionic strength, osmolality, and at low pH.

THP has been the subject of extensive research for its implication in stone formation. However, its exact contribution to urolithiasis remains unclear and the results of various studies have been controversial (Hess 1992). Some studies indicated that THP promoted CaOx and CaP crystallization (Hallson and Rose 1979; Yoshioka et al. 1989). Other studies demonstrated that the macromolecule does not support CaOx crystallization and has no effect on spontaneous precipitation (Yoshioka et al. 1989; Weaver et al. 2009).

Still other studies indicated that THP has no effect on nucleation or growth, but is a potent inhibitor of CaOx crystal aggregation (Hess et al. 1991; Hess et al. 1993). Hess et al. found that the addition of citrate reduced CaOx crystal aggregation by reducing the self-aggregation of THP isolated from stone formers urine. It is important to point out that low citrate or hypocitraturia is common in stone formers and can contribute to crystal
aggregation and stone formation in this fashion. THP activity is controlled by its concentration, urinary osmolality and physicochemical environment of the urine (Scurr and Robertson 1986). For example, at low concentrations, THP has a minor effect on CaOx crystallization yet promotes it at higher concentrations. Also, when ionic strength was increased or the pH lowered the inhibition of CaOx monohydrate crystal aggregation by THP was decreased (Hess et al. 1991). Apparently, at high ionic strength, high THP concentration and low pH, the viscosity of THP increases due to its polymerisation. Several studies have shown that there is no significant difference in the daily urinary excretion of THP between normal subjects and CaOx stone formers (Bichler et al. 1976). This fact led Hess et al. to hypothesize that THP of stone formers is structurally different from that of the healthy subjects (Hess et al. 1991). They showed that THP isolated from the urine of stone formers contained less carbohydrate (mainly sialic acid) than the THP obtained from control subjects (Hess et al. 1995). It has been suggested that the abnormality may be inherited, but sufficient evidence to support this concept is not available at this time.

Studies have also shown differences in sialic acid contents and surface charge between THP from stone formers and normal individuals. Isoelectric focussing (IEF) studies have shown that THP from healthy individuals has a pI value of approximately 3.5, while THP from recurrent stone formers has pI values between 4.5 and 6 and the two exhibit completely different IEF patterns (Schnierle 1995). THP is exclusively produced in the kidneys. Based primarily on studies in rat kidneys, it is agreed that THP is specifically localized in epithelial cells of the thick ascending limbs of the loops of Henle (Hoyer et al. 1979; Bachmann et al. 1990) and is generally not seen in the papillary tubules. When CaOx crystal deposits, the nephroliths, are experimentally induced in rat kidneys, THP is seen in
close association with the crystals, both in the renal cortex as well as papillae (Gokhale et al. 1996a; Gokhale et al. 1996b). However, THP is not seen occluded inside the crystals nor produced by cells other than those lining the limbs of the Henle’s loop (Gokhale et al. 2001). There is no significant biochemical differences in the THP between one secreted by normal rats or rats with CaOx nephroliths. They have similar amino acid composition, carbohydrate contents, molecular weights and rates of urinary excretion. However, THP from nephrolithic rats has slightly less sialic acid contents, 20% of the total carbohydrate in nephrolithic rats vs. 26% in normal rats. In an aggregation assay, both the normal rat THP and nephrolithic rat THP reduced CaOx crystal aggregation in vitro by approximately 47%. Results of these rat model studies led to the conclusions that THP is most likely involved in controlling aggregation and that the major difference between normal and stone formers THP may be their sialic acid contents. However animal studies cannot rule out THP’s role in modulating crystal nucleation or growth. Another rat model study has shown increased expression of THP in kidneys following unilateral ureteric ligation, which caused tubular dilatation (Miyake et al. 1998). The results indicated that THP expression in kidneys may be increased without crystal deposition and that increased expression in nephrolithic kidneys may be a result of crystal associated injury to the renal epithelial cells. Even rat model studies have provided controversial results for THP. One study shows decreased renal expression of THP during CaOx crystal deposition (Marengo et al. 2002) while results of another study show upregulation of the THP gene (Katsuma et al. 2002).

2.5.2.2. Nephrocalcin (NC)

NC is a glycoprotein with a monomeric molecular weight of approximately 14 kDa and has a tendency to self-aggregate into a larger macromolecule and thus can exist as a
dimer, trimer or tetramer with molecular weights of 23-30, 45-48 or 60-68 kDa respectively (Worcester et al. 1992; Nakagawa et al. 1983; Nakagawa et al. 1985; Nakagawa et al. 1987; Nakagawa et al. 1981). NC can also bind to THP in the presence of calcium and magnesium ions. NC can be reversibly dissociated into its monomeric form with incubation in ethylenediaminetetraacetic acid (EDTA) for several days. NC is composed of 110 amino acid residues of which 25% are glutamic and aspartic acid. It contains 2 cysteine and 2 or 3 γ-carboxyglutamic acid (Gla) residues which are suggested to play a significant part in its ability to inhibit CaOx crystallization. Carbohydrate content represents about 10.3% of its weight, with no glucuronic acid and 0.4% sialic acid. Originally purified from human urine, NC has subsequently been isolated from human kidney tissue culture medium, human renal cell carcinoma, kidneys of many vertebrates, mouse renal proximal tubular cells in culture and rat kidney and urine (Coe et al. 1994; Nakagawa et al. 1989). Immunohistochemical techniques have localised NC in the renal epithelial cells of proximal tubules and thick ascending limb of Henle’s loop (Nakagawa et al. 1990). The site of its synthesis has not yet been confirmed by localization of NC mRNA. Daily excretion of NC in human urine is about 5-16 mg (Worcester et al. 1992; Nakagawa et al. 1983). NC was originally regarded as the principal inhibitor of CaOx monohydrate (COM) crystallization in the urine, accounting for approximately 90% of the total urinary crystallization inhibitory activity (Worcester et al. 1992). According to the recent results however, the contribution of this inhibitor is suggested to be limited to only 16% (Worcester et al. 1993). NC is suggested to inhibit nucleation, growth, and aggregation of COM crystals. The fractional inhibition of nucleation due to the presence of NC was shown to be equal to that of urine, (Asplin et al. 1991) suggesting that this inhibitor accounts for the total nucleation inhibitory activity of urine.
However, the amino acid composition and carbohydrate contents of NCs from both the stone formers and normals appeared similar.

2.5.2.2.3. Inter-α-Inhibitor

Inter alpha inhibitor (IαI) are composed of a combination of heavy chains, H1 (60 kDa), H2 (70 kDa), H3 (90 kDa) covalently linked via a CS bridge to a light chain called bikunin (35-45 kDa). The heavy and light chains also exist independently as single molecules. IαI (180-240 kDa) is a heterotrimer consisting of bikunin linked to heavy chains H1 and H2. The macromolecule consisting of bikunin linked to heavy chain H2 is called IαI like inhibitor (IαLI). Bikunin is a broad-spectrum protease inhibitor and an acute-phase reactant. IαI and related proteins have been linked to various pathological conditions such as inflammatory diseases (Witte et al. 1982; Franck and Pederson 1983), cancer (Chawla et al. 1982; Yoshida et al. 1994; Thogersen and Enghild 1995), renal failure (Toki and Sumi 1982) and more recently the urinary stone disease.

Both heavy and light chains have been identified in the urine (Atmani et al. 1993a; Atmani et al. 1993b; Atmani and Khan 1995; Atmani et al. 1996a; Atmani et al. 1994; Suzuki et al. 2001). The average concentration of IαI in the plasma of healthy human subjects is approximately 450 mg/l. It was shown that bikunin isolated from the patients, contained less sialic acid and exhibited less crystallization inhibitory activity than that purified from the urine of healthy subject (Atmani et al. 1994). In a separate study mean urinary bikunin to creatinin ratio was found to be significantly higher in stone formers than in non-stone forming healthy male and female controls (Suzuki et al. 2001). Western analysis showed that a significantly higher proportion of stone patients had a 25kDa bikunin
in their urine in addition to the normal 40kDa species. 25kDa bikunin was similar to the deglycosylated bikunin and was less inhibitory.

With respect to kidney stone formation, Atmani et al. isolated a 35kDa urinary protein, which inhibited growth of CaOx crystals. They named the protein uronic acid rich protein (UAP), because of the high uronic acid content with D-glucuronic and L-iduronic acids being the major constituents (Atmani et al. 1993a). Amino acid composition revealed it to be rich in aspartic and glutamic acid residues, which account for 24% of the total amino acids. No Gla residues were detected. Basic and aromatic amino acids represented 10% and 13%. Carbohydrates accounted for 8.5% of its weight. N-terminal amino acid sequence analysis of human protein demonstrated the homology with 1α1, specifically with bikunin (Atmani et al. 1993b). Later UAP was isolated the from the rat urine (Atmani and Khan 1995) and showed it to have characteristics similar to the human UAP in molecular weight, amino acid composition as well as the crystallization inhibitory activity. In addition, on Western blot analysis, both reacted with an inter-α-trypsin inhibitor antibody. Later, on the basis of bikunin antibody reaction with the UAP in western blot analysis and similarity of the sequence of first 25 N-terminal amino acid residues of UAP being identical to that of bikunin UAP was identified as bikunin (Atmani et al. 1996a). 1α1 proteins have been shown to inhibit CaOx crystallization in vitro (Atmani et al. 1993a; Atmani et al. 1993b; Atmani and Khan 1995; Atmani et al. 1996; Medetognon-Benissan et al. 1999; Kobayashi et al. 1998). The inhibitory activity is confined to the carboxy terminal of the bikunin fragment of 1α1 (Kobayashi et al. 1998).

2.5.2.2.4. Osteopontin
Its apparent molecular weight has been estimated from 44 to 75 kDa depending on the percentage of polyacrylamide gel used. This anomalous migration is assumed to be due to differences in glycosylation and phosphorylation. In addition to its existence as a monomeric form, the protein may also aggregate to form a higher molecular weight entity. Amino acid analysis of rat OP revealed that it contains 319 residues of which 36% are aspartic and glutamic acid (Denhardt and Guo 1993; Prince et al. 1992). It also contains 30 serine, 12 phosphoserine and one phosphothreonine residues.

Osteopontin from all species has high aspartate/asparagine contents accounting for as much as 16-20% of all amino acid residues in the molecule. In addition to bone cells, OPN is present in many epithelial tissues in kidneys, gastrointestinal tract, gall bladder, pancreas, lung, salivary gland and inner ear (Brown et al. 1992). It is also expressed in a variety of other cell types including macrophages (Pollack et al. 1994; Murry et al. 1994), activated T-cells, smooth muscle cells and endothelial cells.

The significantly higher incidence of a single base mutation in the OPN gene has been found in the patients with recurrent or familial nephrolithiasis (Yamate et al. 2000). OPN is intimately involved in both the physiological and pathological mineralisation processes including crystallization in the urine and development of calcific kidney stones support for the CaOx crystallization inhibitory actions of OPN (Langdon et al. 2009) is further strengthened by studies in OPN knockout mice (Wesson et al. 2003). When comparable hyperoxaluria is induced in OPN knockout and wild type mice, knockout mice developed significant intratubular deposition of CaOx crystals while wild type remained free of any crystals. In addition wild type hyperoxaluric mice showed significant increase in OPN expression in their kidneys, indicating a reno-protective role for OPN. Results of one
study show OPN favouring crystallization of COD over COM (Wesson et al. 1998), which may influence the development of kidney stones because renal epithelium is more likely to bind COM crystals than the COD crystals. It appears that structural defects and various post-translational modifications, such as glycosylation and phosphorylation may influence the effect of OPN on crystallization in urine.

2.5.2.2.5. Urinary Prothrombin Fragment –1 (UPTF-1)

This protein is also known as crystal matrix protein (CMP) because it was found selectively associated with CaOx crystals experimentally induced in human urine (Doyle et al. 1991). Molecular weight of this protein was found to be 31 kDa. The amino acid sequence analysis of CMP showed an identity with prothrombin (Stapleton et al. 1993; Stapleton and Ryall 1994; Suzuki et al. 1994), a plasma protein involved in coagulation cascade. In the first 34 amino acid residues, 10 of the glutamic acids are γ-carboxylated. The carbohydrate contents represent 17% of its molecular weight. Suzuki et al. proposed that CMP is the activation peptide of human prothrombin (Suzuki et al. 1994b). By using specific antibodies for prothrombin and F1+2 fragment, Stapleton and Ryall demonstrated (Stapleton and Ryall 1994) that CMP is prothrombin fragment F1 (UPTF-1).

Recent studies have provided evidence that PT gene is expressed in both the human and rat kidneys indicating the possibility of PT biosynthesis in both human and rat kidneys (Grover et al. 2000; Suzuki et al. 1999; Grover et al. 1999). Recent studies using purified urinary proteins have confirmed earlier results and have demonstrated UPTF-1 to be an inhibitor of both crystal growth and aggregation (Ryall et al. 1989). Results of another study where a comparison was being made between the white and black South Africans with regard to urinary crystallization inhibition showed that UPTF-1 is a strong inhibitor of
crystal nucleation (Durrbaum et al. 2001). UPTF-1 from normal black males reduced crystal nucleation by 63.6% as compared to the protein from normal white males that reduced the nucleation by 23.4%.

2.5.2.2.6. Calgranulin (Calprotectin)

Calgranulin is a 28 kDa member of S100 family of calcium binding proteins, which are small, ubiquitous, and acidic proteins involved in normal developmental and structural activities (Zimmer et al. 1995). However, they are also implicated in a number of diseases (Kahn et al. 1982). The protein was recently isolated from human urine (Pillay et al. 1998) at a concentration of 3.5-10 nM. Purified urinary calgranulin inhibited both CaOx crystal growth (44%) and aggregation (50%) in nanomolar range. 28kDa calgranulin was cloned from the human kidney expression library. Western analysis of rat and human kidneys as well as renal epithelial cell lines, BSC-1 and MDCK confirmed its renal presence. Calgranulin is also known as leukocyte antigen L1 and has been identified in circulating neutrophils and 22 monocytes and has bacteriostatic antifungal activities (Steinback et al. 1990). It has also been identified in matrix of infectious or struvite stones (Bennett et al. 1994) and in CaP deposits formed by MDCK cells (Naito et al. 1997; Yasui et al. 1997).

2.5.2.2.7. Albumin

Albumin is one of the most abundant proteins in the urine (Maslamani et al. 2000; Fraij 1989; Boyce and Garvey 1956) and has been detected in the matrix of both urinary stones (Fraij 1989; Boyce and Garvey 1956; Boyce 1968) as well as crystals (Atmani et al.
1998; Atmani and Khan et al. 2002; Atmani et al. 1996a) made in the whole human urine. It
is known to bind to CaOx as well as uric acid crystals (Worcester 1994; Dussol et al. 1995)
but does not inhibit their growth (Worcester 1994). However, it has been shown to inhibit
CaOx crystal aggregation in concentration dependent manner (Edyvane et al. 1986; Hess et
al. 1995; Grover et al. 1998). When immobilized to surfaces and exposed to metastable
solutions albumin promotes crystal nucleation (Cerini et al. 1999; Ebrahimpour et al. 1991).
When dissolved in solution albumin exists either in monomeric or and polymeric form
(Cerini et al. 1999). In metastable CaOx solutions both monomeric and polymeric forms
promote nucleation of CaOx. In addition, nucleation by albumin leads exclusively to the
formation of COD crystals. Urinary albumin purified from healthy subjects contained
significantly more polymeric forms and was a stronger promoter of CaOx nucleation than
albumin from idiopathic calcium stone formers. Promotion of CaOx nucleation and
formation of large number of COD crystals might be protective. Nucleation of large number
of small crystals would allow their easy elimination and decrease CaOx saturation
preventing crystal growth and aggregation and subsequent stone formation. COD crystals
are more common than COM crystals in non-stone formers urine and are generally found in
lesser quantities in stones than COM crystals. In addition crystals present in the urine from
non-stone formers are significantly smaller than those in stone formers urine. Albumin also
exhibits the capacity to bind some of the urinary proteins. Interestingly, urinary proteins that
show great affinity for albumin are also those that are included in the stone matrix. It is
suggested that proteins become a part of stone matrix by binding to the albumin coating
CaOx crystals. It is also suggested that unlike other calcium binding urinary proteins,
albumin promotes nucleation by interacting with calcium through the carboxyl group.
Strong nucleation activity was observed at pH 7 but was totally eliminated at pH 4 when carboxyl groups are no longer ionized. In addition, morphological studies showed CaOx crystals to nucleate through calcium rich face (Cerini et al. 1999).

2.5.2.2.8. CD44

CD44 is a transmembrane protein and the main cell surface receptor for hyaluronan or hyaluronic acid (HA) as well as OPN (Weber et al. 1996). Both CD44 and HA are upregulated during injury and inflammation and are involved in the formation of a cell coat or pericellular matrix on surfaces of proliferating and migrating cells. HA is restricted to the inner medullary interstitium of the normal kidneys. Distal collecting duct cells express both CD44 and HA on apical cell surfaces of the proliferating cells. At confluence however, CD44 is expressed at the basolateral membrane while HA is undetectable. Proliferating cells are receptive to adhesion of CaOx crystals, a property lost when cells become confluent. In addition removal of pericellular matrix by hyaluronidase treatment also results in loss of crystal adhesion property of the proliferating cells (Verhulst et al. 2003; Asselman et al. 2003). Based on these observations it has been proposed that intact epithelium does not bind crystals because of the absence of a pericellular matrix and crystal attachment depends upon the expression of CD44, OPN and HA by the damaged renal epithelial cells (Verkoelen et al. 2000).

2.5.2.2.9. Trefoil Factor1

Chutipongtanate et al. have reported human trefoil factor 1 (TFF1) as CaOx crystal growth inhibitor (Chutipongtanate et al. 2005). It belongs to the trefoil factor family of proteins, is expressed predominately in gastric mucosa, and is synthesized by mucosal
epithelial cells. It has anti-apoptotic and motogenic activities, and its main functions in the gastrointestinal tract are thought to involve maintenance of mucosal integrity and mucosal repair in response to inflammation or injury. TFF1 has been identified previously in human urine using radioimmunoassay and Western blotting. Urinary TFF1 at the concentration of 7 ng/ml inhibited CaOx crystal growth. The significant inhibitory effect was demonstrated after 10 minutes’ incubation and remained significant through the end of the assay (1 hour).

2.5.2.2.10. Model Peptides

A number of studies have been carried out investigating the effect of model peptides on crystallization in vitro. Polyaspartic acids (PolyD) with molecular weights of 8, 12, 15, 37.6 and polyglutamic acids (PolyE) with molecular weight of 13 have been examined. A clear understanding of the crystallization inhibitory mechanisms of various glycoproteins has been the main purpose of these studies. Crystallization of CaOx was induced in vitro in a buffered salt solution containing calcium and oxalate in different ratios and at various supersaturations, in the absence or presence of the polypeptides with pH and ionic strength in the range of normal human urine (Wesson and Worcester 1996; Wesson et al. 2000). In the absence of proteins, CaOx monohydrate was the preferred crystalline form for all calcium to oxalate ratios (Wesson and Worcester 1996; Wesson et al. 2000). The number of CaOx monohydrate crystals increased with increasing oxalate concentrations. The presence of either the Poly D or E produced COD crystals. PolyE was less effective at producing COD than PolyD (Wesson et al. 2000). At a concentration of 800nM and equimolar Ca and Ox concentrations only 20% of the crystals were COD’s. It did however have an effect on COM crystal morphology by producing dumbbell shaped crystals, a morphology common in
human and rat urine. Under similar conditions of supersaturation and Ca and Ox concentrations PolyD, however, favoured the formation of COD requiring very low concentrations <200nM. 12, 15 23 and 37.6 molecular weight PolyD were able to exclusively produce almost all COD’s. Higher CaOx supersaturations required higher amounts of PolyD to cause COD formation. It is concluded that change from COM to COD is the result of inhibition of COM nucleation by protein adsorption onto nascent nuclei. COD is formed to relieve the chemical potential favouring crystallization. The importance of these results with regard to nephrolithiasis lies in the observations that COD crystals are less likely to adhere to the renal epithelium than COM crystals and thus, less likely to be retained in the kidneys and promote the formation of kidney stones (Wesson et al. 2000). Both PolyD and PolyE have also been tested for their effect upon COM crystal growth and adherence to renal epithelial cells in culture. Both proved potent inhibitors of the growth of COM crystals and also blocked the adhesion of COM to BSC-1 cells.

2.5.2.3. Lipids

Even though lipids account for a small proportion of the matrix; 7-14% in bone, 2-6% in dentin, 12-22% in newly mineralised enamel, 9.6% in submandibular salivary gland calculi and 10.2% in supragingival calculi (Wuthier 1981; Anderson 1983; Slomiany et al. 1982; Boskey et al. 1983; Boskey et al. 1981), they play a significant role in calcification. They promote crystal nucleation, modulate growth and aggregation and become incorporated in growing calcifications. The matrix of all stones examined to date, including struvite, uric acid, CaOx and CaP contains lipids (Khan et al. 1988; Khan et al. 1996). The protein to lipid ratio is, however, higher in the matrix of struvite and uric acid stones than in
CaOx and CaP stone matrix. Even though there are no significant differences in types of lipid, the matrix of struvite stones contains more cholesterol, cholesterol ester and triglycerides than the other three stone types. One dimensional thin layer chromatography separated and identified various phospholipids and glycolipids including sphingomyelin (SM), phosphatidyl choline (PC), phosphatidyl ethanolamine (PE), cardiolipin (CL) and trace amounts of phosphatidylserine (PS) in all stone matrices. Occasionally, the stone matrix also contains phosphatidyl inositol (PI), lyso-PC, lyso-phosphatidic acid (PA) and lyso-PE. In all stones glycolipids include gangliosides, D-sphingnosine, and glucocerebrosides. In addition, the struvite stone matrix contains sulfatides and digalacto diglycerides while CaOx and CaP stone matrix contains cerebrosides 1 and 2 and digalacto-diglycerides. All stone matrices contain both complexed and non-complexed lipida. The amount of complexed lipids is highest in CaP and lowest in uric acid stones. Both complexed and noncomplexed lipids contain cholesterol, triglycerides, phospholipids and gangliosides. Both CaOx and CaP crystals induced in the urine contain lipids (Khan et al. 1996). There are no significant differences in either the nature of lipid constituents or the amounts of lipid per gram of crystal between the two types of calcific crystals. Glucocerebrosides are the most common glycolipids and SM the most common phospholipid. Gangliosides are the second most common glycolipid, PC and PE the most common phospholipids. Determinations of lipids in the urine before and after experimental induction of CaOx crystals show that the formation of crystals depletes the urine of its phospholipids indicating its incorporation in the crystal matrix. Almost all the urinary phospholipids become incorporated during the formation of crystals (Khan et al. 1996).